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The Path of Least Resistance: Mechanisms to Reduce Influenza's Sensitivity to Oseltamivir

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The impact of a potential global influenza pandemic calls for the development and delivery of effective antiviral therapeutics [1]. Unfortunately, for all influenza therapeutics widely used in the clinic to date, the virus' high evolutionary capacity has led to the rapid appearance of drug-resistant variants in currently circulating strains [2–4]. Such resistance poses a serious threat to public health, demanding a global effort to generate improved treatment options [5]. The intelligent design of anti-influenza drugs that circumvent resistance requires a molecular-level understanding of the mechanisms involved, such that we can both predict and rationally sidestep such processes.

Current small molecule-based anti-influenza drugs consist of the neuraminidase inhibitors (the focus of this commentary) and the adamantanes, which block the M2 protein ion channel [6]. Neuraminidase is an influenza membrane glycoprotein responsible for cleaving sialic acid from host cell membranes and thereby potentiating viral release [7,8]. Phylogenetic analyses and high-resolution crystal structures of influenza neuraminidase in complex with the enzyme's natural substrate, sialic acid, revealed that residues in direct contact with the substrate are highly conserved among influenza strains (Figure 1A) [9,10]. Information from these high-resolution structures thus provided insight towards the rational design of neuraminidase inhibitors with nanomolar potency and high oral bioactivity [11]. Oseltamivir (Figure 1B) is an optimized compound derived from these studies that is currently a leading anti-influenza drug treatment [5,12,13]. However, oseltamivir displays a C6-pentyloxy group that interacts with a hydrophobic site in neuraminidase, whereas the native substrate sialic acid contains a glycerol moiety at C6 that does not interact significantly with the hydrophobic site [10,14,15]. This distinction has assisted the acquisition of drug-resistant mutations, by enabling neuraminidase variants to exclude oseltamivir from the active site while continuing to process sialic acid with high efficiency in the presence of the drug [14,15]. Alternatively, oseltamivir resistance-conferring mutations have also been observed in hemagglutinin that weaken binding to sialic acid receptors, alleviating the pressure on neuraminidase to cleave sialic acid for virion budding [16].

A commonly observed amino acid substitution in neuraminidase that confers oseltamivir resistance, H275Y, also results in decreased neuraminidase stability and surface expression

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relative to wild-type N1 neuraminidase [17]. The associated fitness costs of the H275Y substitution for influenza prevented this variant from circulating prior to 2008, after which permissive secondary mutations that rescue H275Y neuraminidase surface expression appeared [2, 18, 19]. Significant progress has been made in identifying these compensatory mutations and characterizing their mechanisms of action [18-20]. Beyond the H275Y substitution, it is now known that the I223R/K/T, N295S, and several other amino acid substitutions can also confer oseltamivir resistance, although they simultaneously reduce neuraminidase activity for various reasons [21-24].

Interestingly, reported neuraminidase amino acid substitutions that engender oseltamivir resistance in influenza strains most often occur at active site framework residues, which are residues that interact with functional residues but are not directly involved in the catalytic mechanism of action (Figure 1A) [23, 25]. While mutation of functional residues generally abrogates protein function, mutation of framework residues is usually less detrimental to protein function but can still have significant associated fitness costs. Indeed, prior to the work of Jiang et al. [26], the reported oseltamivir-resistant mutations in neuraminidase had associated fitness costs that often required compensatory, fitness-enhancing mutations for efficient viral propagation.

Although computational methods have had success in specific cases [18], the diverse structural locales of oseltamivir-resistance mutations and the associated permissive secondary mutations question the feasibility of using purely theoretical methods to predict amino acid substitutions that could contribute to antiviral drug resistance. Rationally designing drugs that are less susceptible to antiviral drug resistance mechanisms is instead likely to require highly integrated experimental and theoretical studies. With advances in next-generation sequencing technologies, the field has therefore shifted toward high-throughput screening to systematically identify potential resistance-conferring mutations at single nucleotide resolution. Numerous studies have used a variety of experimental methods to introduce mutations, perform selection, and analyze results [27]. A typical strategy involves random mutagenesis of codons or individual nucleotides of influenza genes of interest (often neuraminidase) in plasmids to generate mutant libraries that are then subjected to selection via passaging in tissue culture in the presence or absence of drug (Figure 2) [28-32]. Viral pools are sequenced before and after selection to determine the relative fitness of each variant. The codon mutagenesis method provides good correlation between true biological replicates (R^2 as high as 0.62) [28] for libraries that include every possible codon mutation for an entire gene ($>10^4$). These studies have produced numerous biomedically significant findings, such as the characterization of the high mutational tolerance of hemagglutinin [28]. Similar methods that generate random mutations at the nucleotide level have also yielded key results, including the identification of several neuraminidase H274Y compensatory mutations and the identification of mutations in the influenza NS1 protein that abolish anti-interferon activity [30, 32].

For both nucleotide and codon mutation libraries generated by mutagenesis, the resultant libraries often contain several mutations per gene, which has the advantage of increasing their throughput and exploration of amino acid space but can complicate interpretation of variant fitness [27, 31, 33-35]. Jiang and colleagues introduce a valuable new experimental

technique to this field that sidesteps the potentially confounding effects of interacting mutations and uneven representation of variants in libraries [26]. Specifically, they adapt the EMPIRIC (Exceedingly Meticulous and Parallel Investigation of Randomized Individual Codons) method [36,37] to study mechanisms of oseltamivir resistance in neuraminidase. EMPIRIC involves the construction of libraries containing all possible single nucleotide mutants in particular regions of a gene (Figure 2). Following a selection step (in this case, influenza replication in the presence or absence of oseltamivir), the relative fitness of each variant is quantified by focused deep sequencing of the small, mutated regions [36,38]. During library generation, EMPIRIC utilizes a cassette ligation strategy that provides equal representation of mutants, maximizing signal during sequencing. Careful construction of these single point mutants enables noise reduction downstream of sequencing by removing reads containing double mutants [36]. EMPIRIC limits the size of the genomic region that is feasible to probe in any given experiment to about 30 base pairs, owing to constraints on sequencing read lengths and base call accuracy [36]. However, exclusively creating single point mutants removes any confounding epistasis or noise from differences in genetic background, contributing to extremely precise measurements of relative fitness and a very strong ($R^2 > 0.9$) correlation between biological replicates. Furthermore, Jiang et al. demonstrate that EMPIRIC is amenable to studying several influenza genomic regions in parallel.

Jiang et al. constructed A/WSN/33 H1N1 neuraminidase libraries containing all possible point mutations for five regions of 30 consecutive bases. The mutated regions were selected to encompass amino acid positions known to contribute to oseltamivir resistance, as well as a control region in a loop far from the neuraminidase active site. After generating comprehensive single mutant libraries for the selected regions, they performed selections as batch competitions in the presence and absence of oseltamivir. Importantly, Jiang et al. are able to identify known oseltamivir-resistant variants such as H275Y and N295S using the EMPIRIC technique. The depth of their mutational scanning also enables the discovery of several novel oseltamivir-resistant variants, including K221N, Y276F, and multiple variants at position 223. Separate fitness analyses on these and other individual variants were performed and the results observed are similar to those from the batch analyses.

As noted above, Jiang and colleagues identify position 223 as a particularly important mutational hotspot for oseltamivir resistance. Indeed, three resistance mutations had been previously identified at this position (resulting in I223R/K/T amino acid substitutions) [23,39]. Jiang et al. discover an additional three using EMPIRIC (I223M/L/V). They further demonstrate that diverse amino acid substitutions at position 223 have an intermediate capacity to attenuate oseltamivir sensitivity. In contrast, variation at other positions, such as 275, results in highly attenuated oseltamivir sensitivity, but only when mutated to one specific codon (i.e., H275Y). Other amino acid substitutions included in the EMPIRIC library do not confer significant oseltamivir resistance. Structural studies show that amino acid residues at position 223 can make direct contact with oseltamivir [39], whereas those at position 275 interfere with the hydrophobic pocket where the pentyloxy group of oseltamivir binds but do not make contact with the native substrate [15]. Cumulatively, these observations suggest that the mutational hotspot characteristics of position 223 relate to its

ability to interact directly with substrate and oseltamivir, which is also the case for analogous mutational hotspots conferring drug resistance in HIV protease [40].

The characterized oseltamivir resistance-conferring mutations in neuraminidase from previous studies and discussed above from this study induce a loss of relative fitness for influenza propagated in the absence of oseltamivir [17,19,21,22,32,39]. The difficulty is that amino acid variation at these positions reduces the enzymatic activity of neuraminidase; a finding that is unsurprising considering that oseltamivir is a substrate analog. Thus, perhaps the most interesting discovery of Jiang et al. is the identification of the oseltamivir-resistant neuraminidase variants Y276F and K221N [26]. Unlike all the other oseltamivir-resistant neuraminidase variants, Y276F and K221N actually enhance influenza fitness both in the absence and the presence of drug. Enzymatic activity studies demonstrate that, unlike the I223M, H275Y, and N295S oseltamivir-resistant variants that decrease neuraminidase activity but also decrease drug affinity, the Y276F and K221N oseltamivir-resistant variants increase neuraminidase activity but do not affect drug affinity. It is apparently via this mechanism that Y276F and K221N manage to provide an adaptive advantage even in the absence of drug, while still conferring drug resistance at low doses of oseltamivir owing to increased substrate processing. This novel mechanism of action represents an intriguing contrast to the view that most drug resistant mutations have an associated fitness cost that can be mitigated by their genetic background. These discoveries highlight the continued importance of developing alternative and innovative influenza treatments, and of continuously deepening our understanding of resistance mechanisms so that we can rationally avoid them.

Overall, the application of EMPIRIC to investigate oseltamivir resistance sets the stage for identifying additional mechanisms of antiviral drug resistance. For instance, Jiang et al. report that mutations known to confer resistance in N2 subtypes are often deleterious in the N1 genetic background on which they focused [26]. An improved understanding of how genetic background influences the acquisition of new amino acid substitutions will be extremely useful for identifying probable resistance mutations in circulating strains. Similarly, the libraries used here consist of single mutants, which allow Jiang et al. to perform precise variant fitness measurements. Once the effects of single mutants have been characterized, it will be interesting to study viruses with combinations of these mutations to extract interactions between mutations that do not affect inhibitor binding but do increase substrate processing with mutations that decrease inhibitor binding and compromise viral fitness. In the future, it will be important to evaluate whether amino acid variants that engender drug resistance via enhancing enzyme activity emerge in vivo, and to assess whether these types of drug resistance mechanisms are important for proteins in other rapidly evolving viruses.

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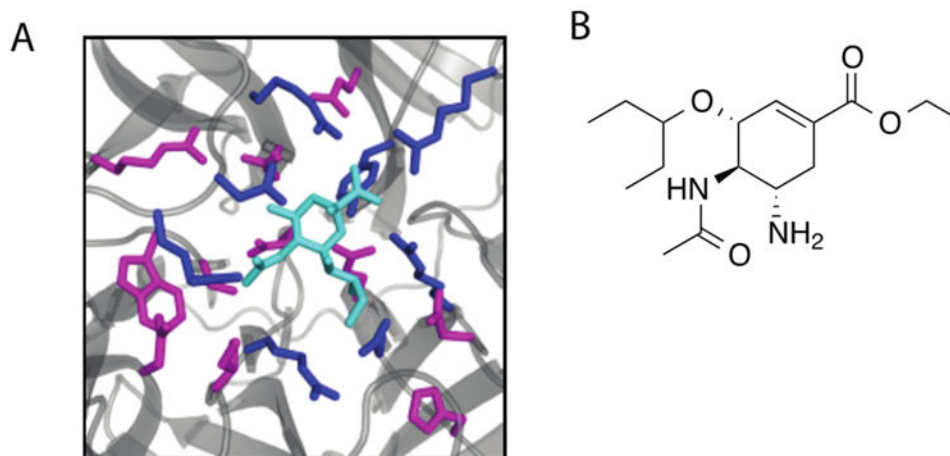


Figure 1.

(A) Structure of N1 neuraminidase with sialic acid bound in the active site. Sialic acid is shown in cyan, functional residues are shown in blue, and framework residues are shown in magenta (PDB 2BAT) [10]. (B) Structure of oseltamivir [12].

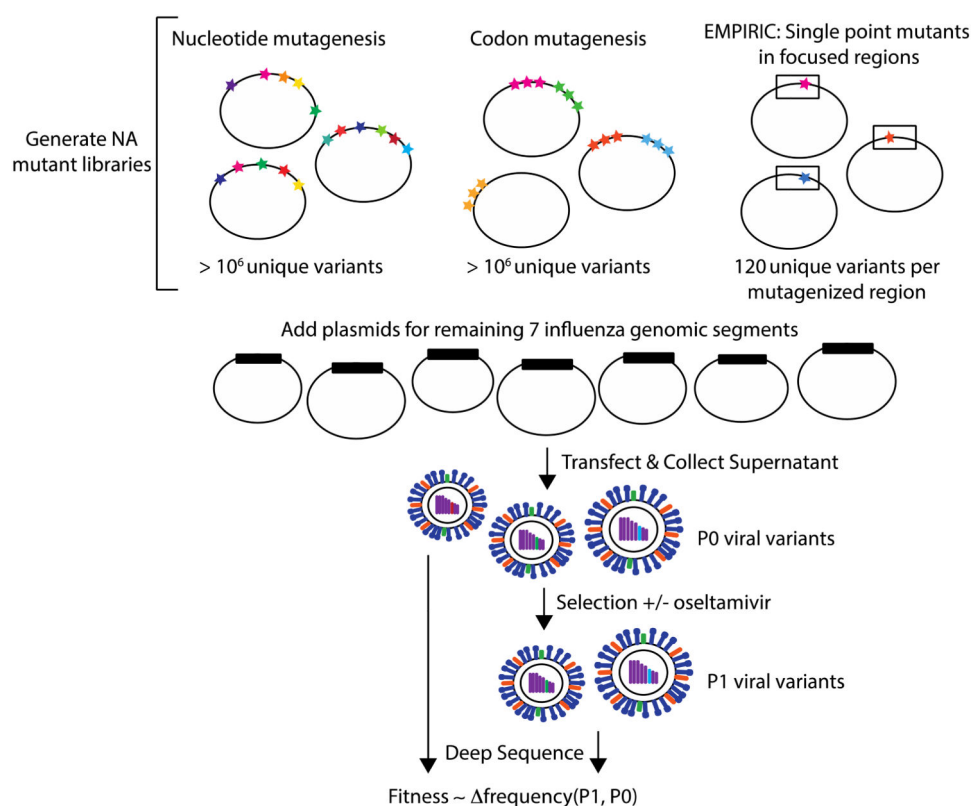


Figure 2. Comparison of methods for constructing neuraminidase mutant libraries

Random nucleotide mutagenesis [27,32,33], random codon mutagenesis [27,28,33], and the EMPIRIC strategy of focused mutagenesis [36,37]. Methods are illustrated in the context of generating mutant libraries to perform selections and quantify variant fitness using deep sequencing.